INVESTIGATION OF FUSARIUM STALK AND KERNEL ROT DISEASES IN FIELD CROPS

A Thesis

by

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ABSTRACT

Fusarium verticillioides is a fungal pathogen of row crops, such as maize and sugarcane, and produces the toxic secondary metabolite, fumonisin B_1 (FB_1). The first topic of this research was aimed to isolate and characterize the Pokkah boeng pathogen through morphological, physiological, and molecular analyses. Pokkah boeng is a serious disease of sugarcane, which can lead to devastating yield losses in crop-producing regions; however, there is still uncertainty about the causal agent of the disease. Sugarcane-colonizing fungi were isolated in Fujian, China and assayed for cell wall degrading enzyme capabilities. Five isolates were identified for further analysis. ITS sequencing revealed that these five strains are *Fusarium*, *Alternaria*, *Phoma*, *Phomopsis*, and *Epicoccum*. The *Fusarium* isolate was further identified as *F. verticillioides* after Calmodulin and EF-1 gene sequencing and microscopic morphology study. Pathogenicity assay confirmed that *F. verticillioides* was directly responsible for disease on sugarcane. Co-inoculation of *F. verticillioides* with other isolated fungi did not lead to a significant difference in disease severity, refuting the idea that other cellulolytic fungi can increase disease severity as an endophyte. For the second topic, a Next-generation sequencing (NGS) experiment was performed to investigate novel genes downstream of MADS-box transcription factors (TFs). With NGS data obtained from *F. verticillioides* wild type and MADS-box TF mutant samples, a computational network-based analysis was used to predict downstream genetic subnetwork modules,associated with FB_1 production. These subnetworks were subsequently

analyzed *in silico*, and five genes hypothesized as putative hub genes were subjected to functional characterization. Modification of RAS GTPase (*SNG3*), cyanate lyase (*SNG4*) and methyltransferase (*SNG5*) resulted in a significant reduction in FB_1 biosynthesis. Conversely, mutation of the 50S ribosome-binding GTPase (*SNG1*) led to an increase in FB_1 production compared to the wild-type. Fitness of the Sng3, Sng4, and Sng5 gene-deletion strains was not adversely affected on V8, defined liquid (DL), and PDA media. With focus on Sng3 and Sng5 strains, a gene expression study was performed to investigate the transcriptional regulatory directionality of neighboring genes in the two subnetworks. Expression of three neighboring genes (FVEG_06215, FVEG_07056, and FVEG_00035) were significantly altered in the Sng3 mutant, furthering the possibility of a hub role for FVEG_02390 in subnetwork A. Gene expression analysis of Subnetwork B in Sng5 demonstrated that FVEG_11168 plays an important role in transcription of neighboring genes (FVEG_07056 and FVEG_06215). Further study into FB_1 regulatory pathways is needed to improve our understanding of the *F. verticillioides* pathogenesis.

DEDICATION

I would like to dedicate this thesis to my mom. Thank you, Mom, for always supporting me through my dreams and endeavors. You have taught me how to be strong and survive through difficult times. Because of you, I have accomplished my goals and continually worked towards my passions. I could not have done this without you. I love you.

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Thanks also go to my friends and colleagues and the department faculty and staff for continually supporting me through the years. When I came to Texas A&M, I lacked confidence and experience but I had a passion for learning. The professors of this department believed in me. They provided me an invaluable academic experience, making me a better student, teacher, and researcher. I could not be any more thankful for their mentorship.

To my lab mates and fellow graduate students, thank you for always offering a shoulder to cry on, a second for laughter and the philosophical engagement needed to make it through this process. Specifically I would like to convey my appreciation to Huan Zhang, my graduate student mentor and friend. Without her technical guidance and continued intellectual support, I could not have so easily accomplished my academic goals.

Finally, I would like to thank my boyfriend, C.J. Graduate school has been enriching but also difficult at times. Thank you for standing by me even when things were tough.

NOMENCLATURE

WT	Wild type
CWDE	Cell-wall degrading enzymes
Dpi	Days – post – inoculation
RT	Room temperature
NGS	Next Generation Sequencing
PKS	Polyketide Synthase
FUM	Fumonisin (-associated genes)
Sng	Subnetwork Gene

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION TO FUSARIUM DISEASES OF FIELD CROPS

The fungal genus *Fusarium* contains a number of plant pathogenic species that have caused extensive economic and agricultural damage throughout history. In particular, species under the *Gibberella fujikuroi* mating population (Anamorphic species in *Fusarium,* section *Liseola,* can infect a variety of field crops, including sorghum, maize, and sugarcane (Kerényi et al. 1999; Bandyopadhyay et al. 2008; Zakaria et al. 2011). Section *Liseola* includes eight known mating populations capable of fertile sexual crosses within each population, subsequently increasing the genetic and morphological variation amongst the species (Leslie 1991; Leslie and Marasas 2008). Many of these fungal species, including *F. verticillioides*, are also producers of toxic secondary metabolites that are dangerous to human and animal health. *F. verticillioides* has been found to produce high concentrations of fumonisin B_1 (FB_1) in maize kernels (Logrieco and Action 2002). Research into the pathogen etiology and genetics is critical for the understanding of row crop diseases and the development of future management practices. The following is a Master's thesis into *Fusarium* diseases of stalk and kernel rot of sugarcane and maize, respectively.

2. IDENTIFICATION AND CHARACTERIZATION OF PATHOGENIC AND ENDOPHYTIC FUNGI ASSOCIATED WITH SUGARCANE IN FUJIAN PROVINCE, CHINA

2.1 Introduction

Sugarcane is an economically important crop worldwide with many agricultural and industrial uses including animal feed, sweeteners, biofuels, and bagasse. Sugarcane is produced in tropical and subtropical countries, including southern regions of People's Republic of China, which is now the fourth largest producer of sugarcane worldwide (Koo and Taylor 2014; Anderson-Sprecher and Wei 2015). China produced over 126 million ton of sugarcane in 2013, but due to recent profit losses, increasing production costs, unfavorable climatic events and disease pressures, production levels are now decreasing up to 7% each year (Anderson-Sprecher and Wei 2015; FAO 2015). Sugarcane is vulnerable to over 100 diseases, which can cause major declines in the annual yield of sugarcane (Matsuoka and Maccheroni 2015). Despite the production decline, the demand for sugarcane still outweighs the annual yield making China the number one importer of sugarcane (Koo and Taylor 2014). It is therefore critical to address the ongoing threats to the sugarcane industry in China and around the world. Pokkah Boeng is a re-emerging disease of sugarcane which has been found recently to cause major yield losses in most sugarcane producing regions, including South Africa, Malaysia, India and China (McFarlane and Rutherford 2005; Singh et al. 2006; Sidique

and Nordahliawate 2007; Vishwakarma et al. 2013; Lin et al. 2014). Pokkah Boeng disease was first described by Walker and Went in 1896, and the name originates from a Javanese for 'malformed top'(Martin et al. 1989). The usual symptoms include red streaking, chlorosis, malformation, and stalk rot. The disease is known to cause 10-38% yield losses in the susceptible sugarcane variety POJ 2878 (Ricaud et al. 2012). Research in India revealed overall increase in disease incidence in all sugarcane varieties from 2007 to 2013, particularly up to 90% infection in variety S224/20 (Moretti 2009; Lin et al. 2014). Historically, the incidence of Pokkah Boeng increase following major rain events or post monsoon season. However, the disease is now present throughout the growing season during both wet and dry periods (Lin et al. 2014).

The causal agent of Pokkah Boeng is still ambiguous and disputed amongst researchers. In older literature, *Fusarium moniliforme* is named as the causal pathogen. However, *Fusarium* classification and nomenclature system has gone through its turbulent transformation, and researchers have not been able to come to a consensus on which *Fusarium* species is the appropriate *forma specialis* (*f.sp.*) for Pokkah Boeng pathogen. Recent studies have suggested *F. sacchari, F. fujikuroi, F. verticillioides, F. andiyazi* and *F. proliferatum* as the causal agents (McFarlane and Rutherford 2005; Singh et al. 2006; Sidique and Nordahliawate 2007; Vishwakarma et al. 2013; Lin et al. 2014). This is in part due to the ambiguity of *Fusarium* taxonomy particularly in the *Liseola* section, which includes *F. verticillioides, F. proliferatum and F. fujikuroi* (Leslie and Summerell 2011). *Gibberella fujikuroi,* or section Liseola, includes eight known mating populations, which are capable of causing disease on a wide range of crops such

as maize, sugarcane and sorghum (Kerényi et al. 1999; Bandyopadhyay et al. 2008; Zakaria et al. 2011). These species are also known to produce a variety of mycotoxins that are detrimental to human and animal health, making the study of these pathogens economically and socially significant. The classification of species falling under section Liseola has gone through both expansion and contraction since the first description of *Fusarium* by Wollenweber and Reinking in 1935 (Wollenweber and Reinking 1935; Waalwijk et al. 1996). With current ambiguity surrounding the causal agent of Pokkah Boeng, one of the important needs is to characterize the etiology of pathogenic *Fusarium* species in sugarcane.

In terms of virulence, we still have a limited understanding of how *Fusarium* pathogens cause disease in sugarcane. When we consider rot diseases in crops, it is reasonable to anticipate that fungal enzymes play a significant role. Many plant pathogenic fungi require cell wall degrading enzymes (CWDE) for successful invasion of plant cell walls and ultimately cause disease (Kubicek et al. 2014). For example, a pectate lyase gene *PL1332* in *Alternaria brassicicola* is required for pathogenicity in *Brassica* (Cho et al. 2015). In *Leptosphaeria manulans,* causal agent of Blackleg of canola, *LmSNF1* is a major regulator of CWDE genes (Feng et al. 2014). CWDE production was downregulated in *LmSNF1* knockout mutants, and strains were avirulent with a reduction in fitness. Furthermore, Jorge and colleagues measured the production of polygalacturonase, pectate lyase and xylanase CWDEs by *F. oxysporum* f. sp. *ciceris* during disease development in chickpea (Jorge et al. 2006). The production of CWDEs was positively correlated to symptom development in leaves and stems, indicating that

CWDEs are important for *F. oxysporum* f. sp. *ciceris* pathogenicity. Sugarcane is a row crop with high recalcitrant lignocellulosic content, therefore, it would be reasonable to hypothesize that the causal agent of Pokkah Boeng produces a high level of cellulolytic cell wall degrading enzymes during plant pathogenesis.

The aim of this study was to isolate and characterize the causal agent of Pokkah Boeng through morphological, physiological, and molecular genetic analyses. We isolated the fungal strains from diseased crops sampled from a sugarcane breeding research field in Fujian, China. Isolates were assessed for the production of cellulase, a prominent cell wall degrading enzyme. In addition, the isolates were inoculated into sugarcane stalks to assess for pathogenicity. Co-inoculations were also performed to gain an understanding of microbial interactions *in situ*. We performed molecular analyses of the isolated strains to determine the identity of Pokkah Boeng causal agent. Our goal was gain a better understanding of this critical disease, as well as how the pathogen is able to produce such devastating outcomes on sugarcane crops.

2.2 Materials and methods

2.2.1 Field sampling

Diseased sugarcane samples were collected at the Sugarcane Research Field at the Fujian Agriculture and Forestry University (FAFU) in China. The area of the FAFU sugarcane research field (approximately 7 hectares) was separated into two 3.5-hectare sections and one of the sections was chosen for sampling. The sugarcane cultivar grown

in the 3.5 hectares sampled is ROC22, a hybrid of *Saccharum officinarum* and *Saccharum spontaneum.* The sampled area was further divided into four different quadrants and an equal number of diseased plants were randomly taken from each quadrant. Diseased plants were observed for the following symptoms: leaf malformation, leaf curling, stalk rot, red streaking, and necroses. Samples were cut, placed in plastic bags, and labeled before returning to the lab for surface sterilization. A total of 74 diseased plants were sampled in June of 2014. Images of the sampled sugarcane are provided in Figure 1.

Figure 1. Characteristic Pokkah Boeng disease symptoms observed in diseased sugarcanes in Fujian, China. A) Red streaking and chlorosis. B) Stalk rot. C) Knife cut and deformed top.

2.2.2 Fungal isolation

Following field sampling, diseased plants were subjected to surface sterilization followed by standard pathogen isolation in the lab (Harborne 1998). Four subsamples of 1-2 cm ²plant tissue were cut out from each sample approximately 2-5 cm away from diseased lesions. These subsamples were placed on sterilized petri dishes for surface sterilization. The surface sterilization process consisted of washing subsamples three times with sterile ddH_2O to remove soil and other surface contaminants. A 10% sodium hypochlorite solution was then applied for 5 minutes on a shaker at 100 RPM. The samples were washed again with sterile ddH_2O before a 70% ETOH solution was applied for 30 seconds. The subsamples were then washed three times with sterile ddH_2O. After surface sterilization, the subsamples were inoculated onto CMI (6% yeast extract, 6% casamino acids, 10% glucose, and 20% agar powder) plates. The CMI plates were then overlayed with CMI supplemented with 0.1% Ampicillin and 0.1% Streptomycin antibiotics. The inoculated plates were placed in an incubator at 25 ℃ with a 12-hour light/dark cycle. Once fungal growth was observed, the mycelia was collected and transferred onto new CMI plates supplemented with 0.1% Ampicillin and 0.1% Streptomycin. Colonies were single spore isolated to maintain purity of the culture (Choi et al. 1999). A total of 137 fungal strains were isolated.

2.2.3 Cellulase/amylase production screening by 3,5-dinitrosalicylic acid (DNS) assay

A total of 87 fungal isolates were screened for cellulase and amylase production using the 3,5-dinitrosalicylic acid (DNS)-based enzyme assay (Percival Zhang et al.

2006; Kim et al. 2014). The fungal isolates were grown in CMI liquid culture with 2.5% glucose and 0.5% wheat bran for 5 days in a shaker at 28 ℃ and 150 RPMs. A 1-ml aliquot was extracted from the top supernatant of the fungal culture and transferred in a 1.5 ml eppendorf tube. The eppendorf tubes were centrifuged at 12,000 RPM for 10 min at room temperature. Approximately 700 µl of the supernatant was transferred into a new 1.5-ml eppendorf tube. For the cellulase assay, 20 µl of a 1% Avicel (Sigma) solution was mixed with 20 µl of fungal supernatant. For the amylase assay, 20 µl of a 1% soluble starch solution was mixed with 20 µl of fungal supernatant. Controls were prepared by mixing sterile ddH_2O and serial dilutions of dextrose. Negative controls with 1% Avicel and 1% soluble starch in sterile ddH_2O were also prepared. The mixtures were then incubated at 45 ℃ for three hours. Following the incubation, a 160-µl DNS solution was added to each tube and incubated at 95-100 ℃ for 20 minutes. The tubes were monitored for a color change, and the ten tubes with most significant color changes were then selected for subsequent characterization. These ten fungal samples were tested again for cellulase production using the DNS assay with three biological replicates per trial and three technical trials. The optical density of each sample was recorded for quantitative analysis using a spectrophotometer at 570 nm.

2.2.4 Molecular and morphological determination of fungal species

To determine the identity of fungal isolates, we amplified genes encoding the ITS region, the Elongation Factor Alpha-1 (ELF) and the calmodulin genes using the primers listed in Supplementary Data. Following the polymerase chain reaction, the amplicons

were sequenced (Gene Technology Lab, Texas A&M), and alignments were performed using BLASTN algorithm. The top sequences with the highest identity were selected and imported into the open source software Jalview Desktop V3 (Waterhouse et al. 2009). Phylogenetic trees were calculated in Jalview using a Neighbor Joining Tree agglomerative clustering method (Saitou and Nei 1987).

Fungal strains were grown on CMI medium for morphological study. All image acquisition was performed using an Olympus BX51 fluorescence microscope outfitted with an Olympus DSU (http://www.olympus-ims.com/en/microscope/) and a Hamamatsu Orca ER camera (http://sales.hamamatsu.com/). The agar block method was used to mount slides for microscopic observation.

2.2.5 Growth assay

The three identified fungal isolates, *Fusarium*, *Phomopsis*, and *Phoma* species, were grown on Complete Media (CMII) plates (Hicks et al. 1997). The fungal spores were harvested and enumerated using a haemocytometer. The concentration of conidia was adjusted to $1x10^8$ per 10-µl sterile ddH_2O and spot inoculated onto CMII, CMI, CMI + 200 mg/ml Congo Red (CR), CMI + 0.01% sodium dodecyl sulfate (SDS), and CMI + 2.5mM hydrogen peroxide (H_2O_2). The diameter of the mycelium was measured after 5 days of growth in a 25 ℃ incubator with a 12-hour light/dark cycle. Three biological replicates per trial in three independently repeated trials were measured. Conidia were collected from the CMII plates after the 5-day incubation period using a borer tube with a 1.3-cm diameter and then counted using a haemocytometer. For the

growth assays in liquid culture, $1x10^8$/ml conidia were inoculated into 50-ml liquid CMI medium. After 5 days at 25℃ in a 150 RPM shaker, fungal mycelia were filtered and dried before weighing on a scale.

2.2.6 Pathogenicity assay

Mature sugarcane stalks were ordered from the Hainan Province, China. The stalks were surface sterilized with 70% ETOH before being cut and separated at the nodes. The sugarcane samples were wounded and then inoculated with 100 μl of a $1x10^8$ spore suspension of *Fusarium, Phomopsis,* and *Phoma* species. Combinatorial pathogenicity assays were also performed by inoculating 100 ml of a 1:1 mixture of $1x10^4$ spore suspension of the following combination of fungi: *Fusarium and Phomopsis, Fusarium and Phoma, Fusarium and Alternaria, Fusarium and Epicoccum nigrum, Phomopsis and Phoma, Phomopsis and Alternaria, Phomopsis and E. nigrum, Phoma and Alternaria, and Phoma and E. nigrum.* The inoculated sugarcane samples were then placed in humidity chambers and incubated for 7 days at approximately 25˚C. Following the incubation, sugarcane samples were cut longitudinally at the wound site to observe the internal rot disease symptoms. Three biological replicates per trial and three technical trials were performed.

2.3 Results

2.3.1 Isolation of putative fungal pathogens of sugarcane

Diseased sugarcane tissues collected from experimental fields in Fuzhou, China were analyzed for the presence of fungal pathogens associated with Pokkah Boeng symptoms. We collected 74 diseased plant samples, and followed standard surface sterilization and pathogen isolation procedures. Not surprisingly, initial culture plates contained a heavy mixture of fungal species and needed further single spore isolation. Ultimately, we isolated 137 fungal strains that showed a wide range of morphological phenotypes on CMI agar plates. To facilitate our screening effort, we sorted fungal strains into groups by visually inspecting on-plate phenotypes, *e.g.*, growth rate, colony morphology, pigmentation, and aerial mycelia abundance. Ultimately, we arrived at 87 isolates as the representative collection of strains for further analysis.

2.3.2 Cell wall degrading enzyme (CWDE) activity screening

We first tested 87 fungal isolates for the ability to produce CWDEs, based on the premise that CWDE is an important characteristic of the Pokkah Boeng pathogen. We cultured fungal isolates in synthetic media with wheat bran as the sole carbon source. Wheat bran has been used to promote cellulase, xylanase, and amylase production in a variety of fungal species (Pandey et al. 1999). Fungal culture extracts were harvested and used to test for the hydrolysis of Avicel (cellulosic fiber) and starch *in vitro* using a standard DNS method as previously described (Percival Zhang et al. 2006; Kim et al.

2014). From our preliminary screening, we identified ten fungal isolates with high enzymatic activity for further testing. We repeated our experiments in biological triplicates, focusing on their ability to hydrolyze Avicel into glucose (Figure 2). To create a standard curve, serial dilutions of Avicel and glucose were prepared and then analyzed using a spectrophotometer. The negative control containing 100% Avicel was used as a blank before analyzing the samples. In this assay, fungal strains 22, 37, and 47 showed significantly higher levels of glucose after the treatment with fungal culture extracts. All other strains showed cellulase production that resulted in detectable glucose conversion. Notably, strains 20 and 27, which were visually diagnosed and predicted as *Fusarium* and *Alternaria* species, respectively, were not one of the highest cellulase producers.

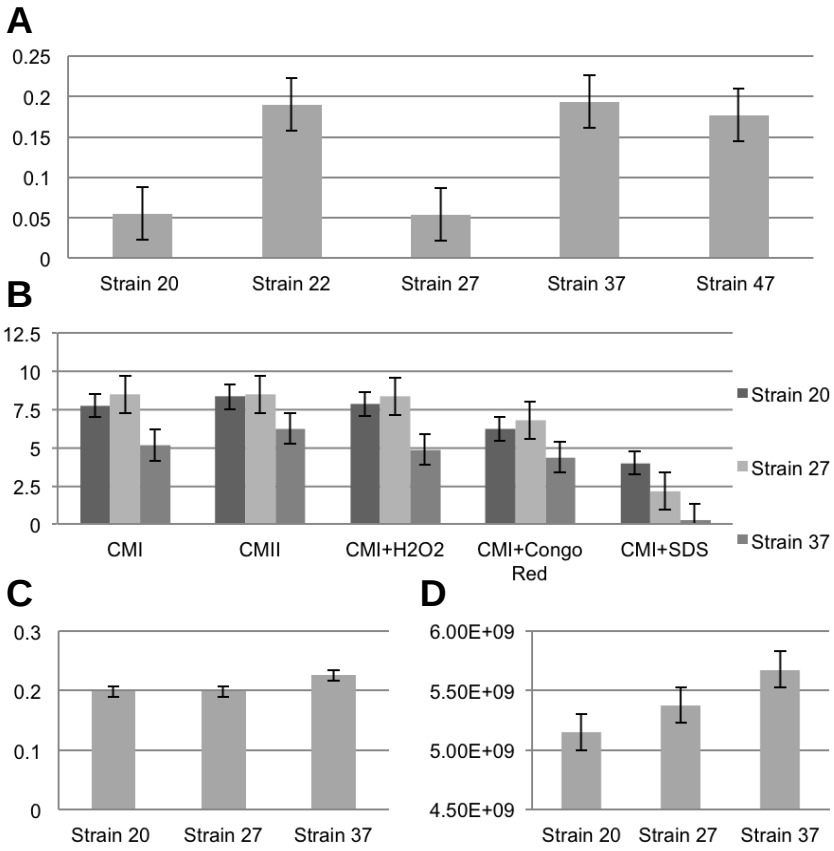


Figure 2. Phenotypic characteristics of fungal strains isolated from diseased sugarcane. A) Average glucose conversion from Avicel by the fungal isolates Strain 20 (Fusarium), Strain 22 (Alternaria), Strain 27 (Phomopsis), Strain 37 (Phoma), and Strain 47 (Epicoccum), B) Growth rate analysis of Strain 20 (Fusarium), Strain 27 (Phomopsis) and Strain 37 (Phoma) on different synthetic media. C) Average weight of fungal biomass of Strain 20 (Fusarium), Strain 27 (Phomopsis), and Strain 37 (Phoma) in CMI Broth. D) Production of conidia by Strain 27 (Phomopsis), Strain 20 (Fusarium), and Strain 37 (Phoma) in CMII media.

2.3.3 Molecular and morphological species determination

Once we selected five sugarcane-associated fungi for further study, we performed DNA sequencing and microscopy for species identification. We isolated

genomic DNA samples from five fungal strains and sequenced the amplified ITS regions (Figure 3). We determined that 475-bp ITS from strain 20 (GenBank KU508286) shared 100% match with *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) UFMGCB1229 (GenBank FJ605250), *F. fujikuroi* (teleomorph *G. fujikuroi*) GX41 (GenBank KJ000435) and *Fusarium* sp. ASR-126 (GenBank GU973689). However, it is important to note that we found more than 20 sequences showing 100% alignments, and all were *Fusarium* species (data not shown). The 545-bp ITS from strain 22 (GenBank KU508289) best matched those of *Alternaria alternata* 38-8-A (GenBank KR817678), *Alternaria* sp BAB-5085 (GenBank KT186138) or *Alternaria* sp 311a (GenBank KM507780). We also determined strain 27 (GenBank KU508290) as *Phomopsis* sp. C3P42D (GenBank JQ936281), *Diaporthe endophytica* CBS133811 (GenBank NR_111847) or *D. longicolla* CMT38 (GenBank JQ754027). Strain 37 (GenBank KU508291) was highly related to *Phoma* sp FJ-130109 (GenBank KC961262) or *Epicoccum sorghinum* A1S6-11 (GenBank KJ767080). Lastly, strain 47 (GenBank KU508292) was determined as *Epicoccum nigrum*.

The fact that several *Fusarium* species have been suspected as the causal agent of Pokkah Boeng prompted us to perform additional DNA analyses of strain 20 with calmodulin (GenBank KU508287) and ELF-1 genes (GenBank KU508288) (McFarlane and Rutherford 2005; Singh et al. 2006; Sidique and Nordahliawate 2007; Vishwakarma et al. 2013; Lin et al. 2014). Sequence analysis showed that ELF-1 gene matched *F. verticillioides* while calmodulin gene showed highest similarity with *F. proliferatum.*

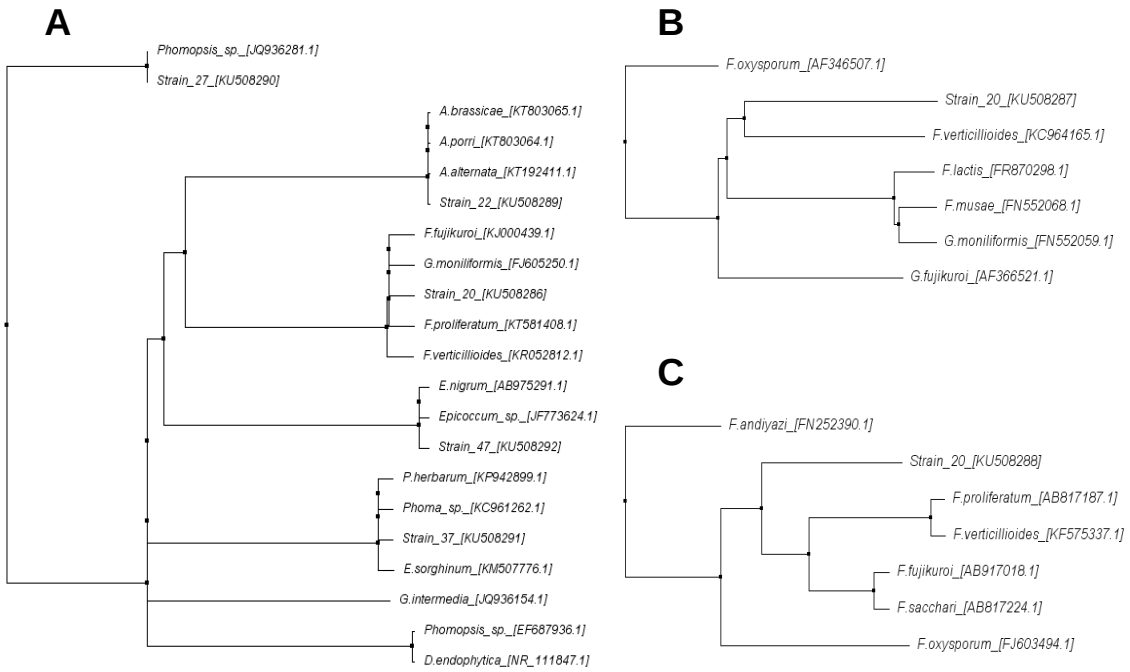


Figure 3. Phylogenetic analyses of fungal strains isolated from diseased sugarcanes. A) Five fungal strains were analyzed based on ITS DNA sequence. B) Fusarium strain analysis with Translation Elongation Factor 1-alpha DNA sequence. C) Fusarium strain analysis with Calmodulin DNA sequence.

Subsequently, we gathered morphological images that can help our classification effort. We concluded that the five isolates are members of the Phylum Ascomycota or Deuteromycota. Hyphae possessed simple septa and each of the fungi produced asexual conidia. In particular, strain 20, the *Fusarium* species, produced microconidia that lacked septa and had monophialic conidiophores (Figure 4). This species description is consistent with that of *F. verticillioides* (teleomorph *G. moniliformis*), a fungus that is very closely related to *F. proliferatum* (Leslie et al. 2008).

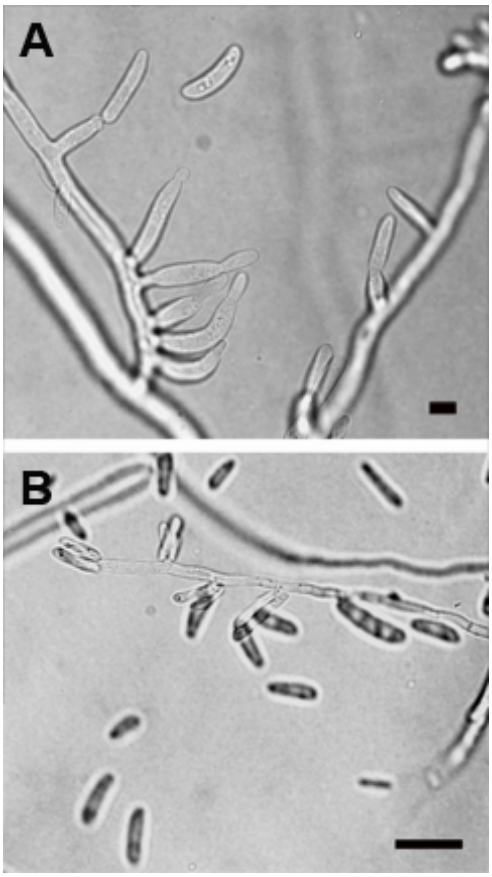


Figure 4. Microscopic observation of Strain 20 *Fusarium* species. A) Hyphae possess simple septa and produce conidiogenous monophialide cells (error bar = 10 mm). B)Microconidia are oval to club shaped and lacks septa (error bar = 100 mm).

2.3.4 Vegetative growth on solid media and in liquid culture

Our species identification was followed by vegetative growth assays, particularly focusing on fungal tolerance to stress agents. Fungal plant pathogens are exposed to a wide range of stress agents both from the ambient environment and *in planta*, which can affect virulence and fitness. Congo Red, SDS, and hydrogen peroxide have been used in

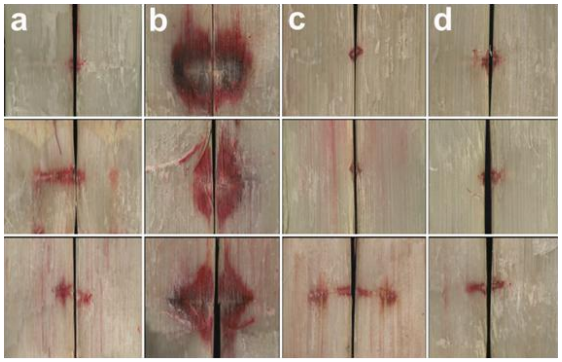
previous studies to imitate oxidative stress on fungal cells (Roncero and Durán 1985; Gerik et al. 2008). Here we focused on *Fusarium*, *Phomopsis*, and *Phoma* isolates since these fungal species are recognized as aggressive phytopathogens in crops (Figure 2). When we measured the colony diameter on agar plates, the *Fusarium* isolate grew at approximately equal rates in comparison to the *Phomopsis* strain but grew significantly faster than *Phoma* on the CMI and CMI+H_2O_2 media. The addition of Congo Red inhibited the growth of *Fusarium* but did not significantly affect the growth of *Phoma* or *Phomopsis* strains. Interestingly, the addition of SDS drastically inhibited the growth of *Phomopsis* and *Phoma*. While the growth of *Fusarium* strain was negatively affected by SDS, the fungus exhibited a drastically vigorous growth on CMI+SDS in comparison to *Phoma* and *Phomopsis* strains. Other than the growth rate, the addition of the H_2O_2, SDS, or Congo Red did not have any detrimental effect on the overall phenotype of fungal strains when visually inspected.

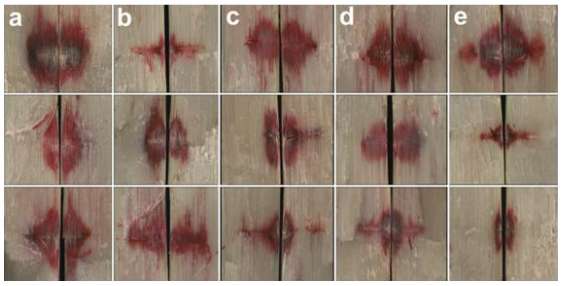
In CMI liquid medium, *Phomopsis* produced the highest mycelial mass followed by *Phoma* and *Fusarium*. The only significant difference in the production of mycelia was between the *Phomopsis* and *Fusarium* isolate. After collecting conidia, it was found that *Phoma* produced the highest number of conidia, followed by *Phomopsis* and *Fusarium* strains. The production of conidia was significant in comparison to *Fusarium but* not in comparison to *Phomopsis. Fusarium* strain produced both macroconidia and microconidia in CMI culture medium.

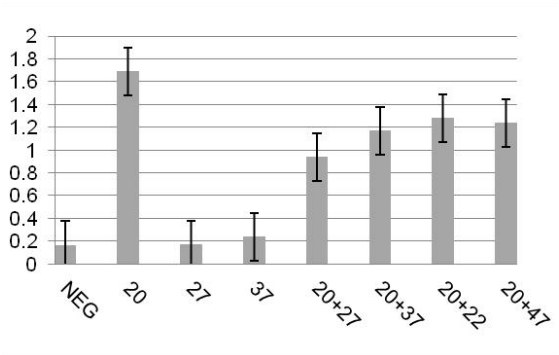
2.3.5 Pathogenicity analysis

The three pathogenic fungi, *Phoma, Phomopsis,* and *Fusarium,* were independently inoculated into mature sugarcane stalks (Figure 5). *Phoma* and *Phomopsis* strains produced little to no lesions and were comparable to the negative water control. However, inoculation of the *Fusarium* led to extensive tissue maceration and rot. The experiment was conducted in three biological replicates, and showed consistent outcomes. This outcome, along with our morphological and phylogenetic studies, strongly suggests that *F. verticillioides* is a key pathogen of sugarcane fields in Fujian, China.

The next question we asked was whether four other fungal isolates could influence the severity of lesions caused by *F. verticillioides* when coinoculated into sugarcane stalks. In literature, *Phomopsis*, *Phoma*, *Alternaria,* and *Epicoccum* are recognized as pathogens, saprophytes, or endophytes. More importantly, we have determined that these isolates can produce a high level of cell wall degrading enzymes under laboratory conditions (Figure 2). Therefore, *F. verticillioides* isolate was co-inoculated into sugarcane stalks with either *Phoma, Phomopsis, Epicoccum,* or *Alternaria* (Figure 5B). Results showed that the individual *F. verticillioides* inoculation led to a significantly larger lesion than the co-inoculation of *F. verticillioides* and *Phomopsis* based on a 95% confidence interval. We did not find significant difference between the individual inoculation of *F. verticillioides and* other co-inoculated samples. In addition, analysis of variance (ANOVA) revealed no significant difference between the co-inoculations and the individual inoculation of *F. verticillioides.*







ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3.339	4	0.834	1.051	**0.395**	2.649
Within Groups	27	34	0.794			
Total	30.34	38				

Figure 5. Pathogenicity assay. Fungal isolates were inoculated into sugarcane stalk and after 7-day incubation cut longitudinally at the wound site to assess disease severity. A) a: negative control, b: Strain 20 (Fusarium), c: Strain 27 (Phomopsis), d: Strain 37 (Phoma), 601 B) a: Strain 20 (Fusarium), b: Strain 20 (Fusarium) and Strain 27 (Phomopsis), c: Strain 602 20 (Fusarium) and Strain 37 (Phoma), d: Strain 20 (Fusarium) and Strain 22 (Alternaria), e: Strain 20 (Fusarium) and Strain 47 (Epicoccum). C) Average lesion size produced by fungal strains in sugarcane stock when inoculated independently and in combination. D) ANOVA statistic to compare average lesion size in co-inoculation study. The evidence does not support a significant difference in the lesion size of strain 20 (*Fusarium)* compared to the coinoculation of strain 20 (*Fusarium)* with other sugarcane-colonizing fungi.

2.4 Discussion

Sugarcane is produced throughout tropical and subtropical regions of the world. Pokkah Boeng is a disease of sugarcane that can lead to 38% yield losses and 90% infection rate in susceptible varieties (Ricaud et al. 2012; Vishwakarma et al. 2013; Lin et al. 2014). Sugarcane producing regions are experiencing an increase in severity and incidence of Pokkah Boeng throughout the growing season (Lin et al. 2014). The aim of this study was to identify the causal agent of Pokkah boeng by characterizing samples collected in Fujian, China. Proper identification of the causal agent for Pokkah boeng is important to gain clearer understanding of the disease and the subsequent development of breeding and management practices to minimize future losses.

F. moniliforme species complex under section *Liseola* has been reclassified since the 1990s, and there is still on-going discussion amongst researchers to clarify the nomenclature. Recently, several species of *Fusarium* have been reported as the causal agents of Pokkah Boeng, including *F. sacchari*, *F. fujikuroi*, *F. verticillioides*, *F. andiyazi* and *F. proliferatum* (McFarlane and Rutherford 2005; Singh et al. 2006; Sidique and Nordahliawate 2007; Vishwakarma et al. 2013; Lin et al. 2014). Classification of *Fusarium* species relies on morphological characteristics along with phylogenetic and molecular techniques (O'Donnell et al. 1998; Moretti 2009). In this study, we used a combination of morphological, molecular, and phylogenetic techniques to identify the isolated causal agent of Pokkah Boeng disease. The isolate was found to possess morphological characteristics consistent with *F. verticillioides*, however

phylogenetic analysis revealed 100% identity with both *F. verticillioides* and *F. proliferatum.* Here, we are concluding that the causal agent of Pokkah Boeng that we isolated from Fujian, China is *F. verticillioides.* However, given the phylogenetic similarities between these two species, there is a need for broader and deeper investigation into the population in southern China to arrive at a more definitive conclusion.

Cell wall degrading enzymes (CWDE) are known as key virulence factors associated with a number of pathogenic microbes (Kubicek et al. 2014). *F. graminearum,* a species closely related to *F. proliferatum and F. verticillioides,* encodes at least 30 diverse xylanase genes that are known to be involved in head scab disease of wheat (Mary Wanjiru et al. 2002; Hatsch et al. 2006). When initiating our screening for the putative pathogen of Pokkah Boeng our premise was that high CWDE production would be associated with pathogenesis. Our study showed that the isolated *F. verticillioides* was determined as one of the highest producers of cellulolytic enzymes from the pool of 87 fungal isolates. Genomic analysis of carbohydrate-active enzymes (CAZymes) across a wide range of fungi revealed that *F. verticillioides* has the highest number of CAZymes (Zhao et al. 2013). *F. verticillioides* genome encodes for over 800 CAZymes, and approximately 28 different types of cellulase (Zhao et al. 2013; Kubicek et al. 2014). However, the fact that the isolated *F. verticillioides* was not the highest CWDE producer suggests that there are likely other virulence factors contributing to the infection of sugarcane. Another explanation would be a difference in the production of cellulolytic enzymes *in vitro* versus *in vivo*. For example, CWDE production in *Botrytis*

cindered is variable dependent on the degree of infection and pathogen localization in the host (Verhoeff and Warren 1972). Furthermore, Zhao et al (2013) found that *Rhizoctonia solani* produces polygalacturonase and polymethylgalacturonase at higher concentrations *in vitro*, while the activity of β-glucosidase and carboxymethyl cellulase is highest *in vivo*. Further analysis of cellulase production by the isolated *Fusarium* isolate *in vivo* will need to be carried out in order to make additional conclusions.

During our screening, we discovered that four fungal isolates other than the pathogenic *F. verticillioides*, *i.e*., *Alternaria, Phoma, Phomopsis* and *Epicoccum*, exhibited high levels of cellulase production, albeit the fact that none were able to independently cause disease in sugarcane. This outcome was unexpected since *Alternaria, Phoma and Phomopsis* species are well recognized as aggressive plant pathogens, causing lesions and rots in a variety of crops (Agrios 2005). Subsequently, we asked whether these fungal isolates play supplementary role in Pokkah Boeng pathogenesis, perhaps enhancing the aggressiveness of *F. verticillioides* during colonization and rot. However, our co-inoculation experiments showed that these four species have no impact on the severity of the disease and are likely endophytic co-inhabitants in sugarcane.

Recently, Shrestha et al (2015) isolated over one hundred fungi from sugarcane and silvergrass (*Miscanthus*) in the United States to study fungal species for enzymatic activity, which included *Alternaria, Phoma, Epicoccum* and *Fusarium* species. Amongst the tested, *E. nigrum* was one of the top producers of endocellulase, glucosidase, and xylanase, whereas *F. proliferatum* strain isolated from silvergrass was found to be only a

moderate producer of CWDEs. However, it is unclear whether Shrestha and colleagues tested these fungal isolates for pathogenicity. Favaro et al (2011) isolated 112 *Epicoccum* strains from sugarcane in Brazil, separated them into two genotypic groups (Group I – *E. nigrum;* Group II – novel *Epicoccum* species) and subsequently analyzed these strains for CWDE production. Over 90% of isolates in Group I were capable of producing polygalacturonase, pectin lyase and lipases, while the isolates classified in Group II were less capable of producing the CWDEs. One symbiotic isolate, *E. nigrum* isolate P16, was determined to induce root growth in sugarcane and inhibit the growth of the pathogens *in vitro*, in particular *F. verticillioides* (de Lima Favaro et al. 2011; de Lima Favaro et al. 2012). We questioned whether our *E. nigrum* strain possessed antifungal properties, but the *E. nigrum* strain did not exhibit any inhibitory effect on the pathogenic *F. verticillioides* strain isolated in this study (data not shown).

With the evidence supporting the role of *E. nigrum* as a beneficial endophyte, it raises a interesting possibility that *E. nigrum* uses its arsenal of CWDE during a saprophytic shift the in the fungal lifestyle (de Lima Favaro et al. 2012). This outcome coupled with the CWDE producing capabilities of the fungus further provides an insight into the diversity of *E. nigrum* throughout sugarcane growing regions of the world, as well as symbiotic interactions of this particular endophyte with pathogenic fungi. There are several commercial products containing fungal biocontrol agents, which can reduce disease severity directly through the production of antifungal compounds or by inducing the host plant defense responses (Schulz et al. 2002; Butts and Krysan 2012). For example, *Acremonium* isolated from date palm reduced wilt symptoms by up to 87%

when antagonized with *F. albedinis in vivo* (El-Deeb and Arab 2013). In contrast, there is also evidence that the interaction between some fungi and other pathogens may lead to an increase in disease severity. Ridout and Newcombe (2015) co-inoculated blight pathogen *Dothistroma* with six different endophytic isolates onto pine needle. Four fungi, *Sydowia polyspora, Bionectria ochroleuca, Penicillium raistrickii,* and a culturable species of *Elytroderma,* increased the disease severity of *Dothistroma* by 4.7%, 4.2%, 3.6%, and 2.5%, respectively. In our current study, we co-inoculated *F. verticillioides* with *Phoma, Phomopsis, Alternaria,* and *Epicoccum* into the sugarcane hybrid. Given the evidence for an arsenal of CWDEs in the *Epicoccum, Phoma,* and *Alternaria* strains, and the literature supporting the role of *Epicoccum nigrum* as a potential biocontrol, we hypothesized that interactions between fungi would lead to a difference in pathogenesis of the *Fusarium* pathogen. However, after analyzing the lesion size amongst each of the replicates, no significant difference was found in the disease severity.

3. SUBNETWORK MODULE ANALYSIS OF MADS-BOX TRANSCRIPTION FACTORS (TF) IN FUSARIUM VERTICILLIOIDES

3.1 Introduction

Fusarium verticillioides is an economically important maize pathogen known to cause stalk and kernel rot, and has been found to produce high concentrations of Fumonisin B_1 (FB_1) in maize kernels (Logrieco and Action 2002). Animal feed contaminated with FB_1 has been found to cause leukoencephalomalacia in horses (ELEM), pulmonary oedema and hepatic syndrome in swine (PPE), poor performance in poultry, and alteration in hepatic and immune function in cattle (Bennett and Klich 2003). Furthermore, ingestion of fumonisin contaminated corn-based food products has been strongly linked to esophageal cancer in humans (Thiel et al. 1992; Norred and Voss 1994). Elucidation of the signaling pathways associated with mycotoxin biosynthesis is critical for improving our understanding of *Fusarium* secondary metabolism and for facilitating innovative approaches to control diseases of *F. verticillioides* in maize and other field crops.

The complexities of secondary metabolism pathways have continued to puzzle researchers, yet strides have been made towards the discovery of key players in FB_1 biosynthesis and regulation. Researchers have identified the *FUM* gene cluster, which includes approximately 10 genes clustered near the *FUM1* locus that are responsible for

the enzymatic biosynthesis of FB_1 (Proctor et al. 2003; Alexander et al. 2009; Picot et al. 2010). *FUM1* encodes a polyketide synthase, a common enzyme that catalyzes several classes of polyketides involved in the secondary metabolism of plants, animals, and microorganisms (Hopwood and Khosla 1992; Castoe et al. 2007). MADS-box TFs, along with several additional genes, have been found to modulate FB_1 production and *FUM/PKS* gene expression in *F. verticillioides* (Picot et al. 2010; Ortiz and Shim 2013). Disruption of *MADS1, AREA, FvFE1* and *FUM21* all exhibited a significant impact on downstream *FUM* gene expression and resulted in reduced or eliminated FB_1 production in mutants (Brown et al. 2007; Kim and Woloshuk 2008; Myung et al. 2009). Differential expression of *FUM* genes and, in effect, changes to fumonisin production can also be impacted by various environmental factors such as water availability, source of nitrogen, light conditions and/or pH levels (Shim and Woloshuk 1999; Fanelli et al. 2012; Fanelli et al. 2013). Given that biological signaling and biosynthesis pathways are often formed of intricate web of genes acting synergistically, we can expect that networks of genes involved in fumonisin production likely occur downstream of these key regulatory genes.

MADS-box TFs play a number of pivotal roles in the co-regulation of genes, signal transduction and developmental functions, such as floral development in *Arabidopsis*, co-regulation of the proto-oncogene *c-fos* in animals, and pheromone production in *Saccharomyces cerevisiae* (Shore and Sharrocks 1995; Gramzow et al. 2010). They are defined based on primary sequence similarity and possess a highly conserved motif across a wide range of eukaryotes. The name, MADS, originates from

the first four novel proteins identified: *MCM1*, *AGAMOUS*, *DERCIENS*, and *SRF1*. The MADS-box Serum Response Factor 1 (*SRF1*) is a homodimer-specific nuclear resident transcription factor activated by serine phosphorylation (Messenguy and Dubois 2003). The consensus binding sequence of *SRF1* is $CC(A/T)_6GG$ or the CArG box. Two MADS-box TFs, namely *Mads1* and *Mads2*, were recently characterized for their role in secondary metabolite production and sexual reproduction in *F. verticillioides* (Ortiz and Shim 2013). Knockout mutants were constructed to analyze the phenotypic and biological effect of these genes. Results revealed that the MADS-box TFs have a significant impact on fumonisin production, with a 50% FB_1 reduction in the *Mads1* knockout mutant compared to that of the wild type (WT). Real time quantitative RT-PCR was used to analyze the expression of 15 polyketide synthase (PKS) genes in the WT and *Mads1* strains. Expression levels in *Mads1* were equal to or greater than the WT at 6-days post-inoculation (dpi), and began to decrease as time passed. At 10-dpi, the relative quantity of 14 of the PKS gene transcripts were reduced to at least half in *Mads1* compared to the WT.

Continuing the effort to characterize MADS-box TF regulatory pathways in *F. verticillioides*, a next-generation sequencing (NGS) experiment was performed to compare the expression pattern of genetic networks downstream of MADS-box TFs and as a result, identify novel genes with an impact on secondary metabolite production in *F. verticillioides.* NGS has become a widely available approach to generate massive, high-throughput datasets, and has led to an improved understanding of genomes, transcriptomes, and interactomes in biological systems (Schuster 2007; Shendure and Ji

2008). However, many in the scientific community are now contemplating how best to analyze the large NGS datasets and, in turn, extract meaningful biological information (Ozsolak and Milos 2011). The following study is aimed at developing systematic approaches to investigating the interrelationship between coexpressed genes rather than simply compare quantitative transcript abundance as a measure of significance. Conceptually, the co-expression network analysis used in this study is analogous to traditional differential gene expression analysis, except that the approach investigates the system-level changes across correlated genes rather than simply focusing on individual gene expression. Genes, as well as proteins and other cellular components, do not perform their functions in isolation or in linear pathways but rather influence others to form complex, scale-free networks, and often a 'network of networks', to determine cellular behavior (Albert 2005). Similar network-based comparative analyses have been applied in medical microbiological experiments, however, these approaches are still relatively novel in the field of phytopathology (Kim et al. 2015a; Kim et al. 2015b). Furthermore, few experiments have been performed to verify modeling and network predictions *in vivo*. Application of computational analyses will allow us to discover functional subnetworks and key genes previously unknown in fungal secondary metabolism and FB_1 production.

3.2 Materials and methods

3.2.1 Fungal strains, culture media, and growth conditions

Wild type (WT) *F. verticillioides* strain 7600 (Fungal Genetics Stock Center, Kansas City, MO, USA) as well as mutant strains generated in this study (Table 1) were stored in 30% (v/v) glycerol at -80 ℃ (Shim and Woloshuk 2001; Sagaram and Shim 2007). Conidia for use as inoculum were generated by growing the fungus on V8 agar plates for 7–10 days at 25 ℃. Conidia were then harvested in sterile water and quantified using a haemocytometer (Stevens and Ammirati 1981). For genomic DNA extraction, strains were grown in YEPD liquid medium for 2-3 days at 25 ℃ on a150-RPM rotary shaker (Shim and Woloshuk 2001). Tissue for RNA extraction was grown in defined liquid (DL) medium at 25 ℃ on a 150-RPM rotary shaker, and harvested at 5- and 7-dpi (Ortiz and Shim 2013).

Strain	Description	Mating type	Genotype
7600	F. verticillioides wild-type strain	MAT1-1	FVEG_00035; FVEG_07056; FVEG_02390; FVEG_07804; FVEG_11168*
Mads1	Deletion of MADS1 in 7600	MAT1-1	ΔMADS1 :: HPH
Sng1	Deletion of FVEG_00035 in 7600	MAT1-1	ΔFVEG_00035 :: HPH†
Sng2	Deletion of FVEG_07056 in 7600	MAT1-1	ΔFVEG_07056 :: HPH†
Sng3	Deletion of FVEG_02390 in 7600	MAT1-1	ΔFVEG_02390 :: HPH†
Sng4	Deletion of FVEG_07804 in 7600	MAT1-1	ΔFVEG_07804 :: HPH†
Sng5	Deletion of FVEG_11168 in 7600	MAT1-1	ΔFVEG_11168 :: HPH†

*Strain Source: Fungal Genetics Center
†Strains generated in this study

Table 1. Fungal strains used in this study.

3.2.2. Preprocessing, computational analysis and subnetwork prediction

The WT strain and MADS-box TF disruption mutant (*Mads1*) from a previous study (Ortiz and Shim 2013) were inoculated in YEPD liquid broth and incubated at 25°C and 150-RPM. The fungal tissue was harvested at 7-dpi, and 0.3 grams of mycelia was inoculated into synthetic Defined Liquid (DL) medium (Shim and Woloshuk 2001) to promote FB_1 production. Total RNA was extracted from five biological replicates of the WT and *Mads1* strains at 5- and 7-dpi using TRIzol reagent (Invitrogen Life Technologies) according to manufacturer's protocols. The RNA samples were preprocessed and sequenced with Illumina HiSeq2000 following the standard protocol at Texas A&M AgriLife Genomics and Bioinformatics Services (College Station, Texas).

Following QA/QC procedures and library preparation, sequence reads were aligned to the reference *F. verticillioides* strain 7600 genome downloaded from Broad Institute (http://www.broadinstitute.org.) in collaboration with researchers in the Electrical & Computer Engineering department at Texas A&M University (Kim 2015; Kim et al. 2015b). In brief, aligned genes were filtered, unexpressed genes were removed, and differentially expressed genes were normalized based on gene length, read counts, and *Mads1* β-tubulin expression. Five thresholds (i.e. 0.90, 0.91, 0.92, 0.93, and 0.94) of significant partial correlation were used to construct co-expression networks and predict gene interaction. Subnetworks were then identified using a seed-and-extend approach, in which the top 1% of differentially expressed genes were selected as seed genes and verified by t-test statistics. Coorelated genes were systematically expanded from seed genes, creating edges, and forming complex, scale-free subnetworks. Subnetwork robustness was assessed based on the following six criteria: i) probabilistic impact, ii) relatively differentially correlated between two strains (WT vs. *Mads1* mutant), iii) relatively more connected in the given module, iv) relatively highly expressed in WT, v) orthologous to known pathogenic genes of other fungi, and vi) annotated to significant GO terms with other member genes. During this process, subnetwork activity levels were measured between the two phenotypes using a log likelihood ratio (LLR). Potential pathogenicity genes that demonstrated not only strong association with fungal secondary metabolism, but also displayed significantly differentiated activity levels between the two phenotypes (WT vs. *Mads1*) were selected from each subnetwork. Two

optimal predicted subnetworks were then chosen for subsequent analysis *in silico*, followed by functional characterization (Figure 6).

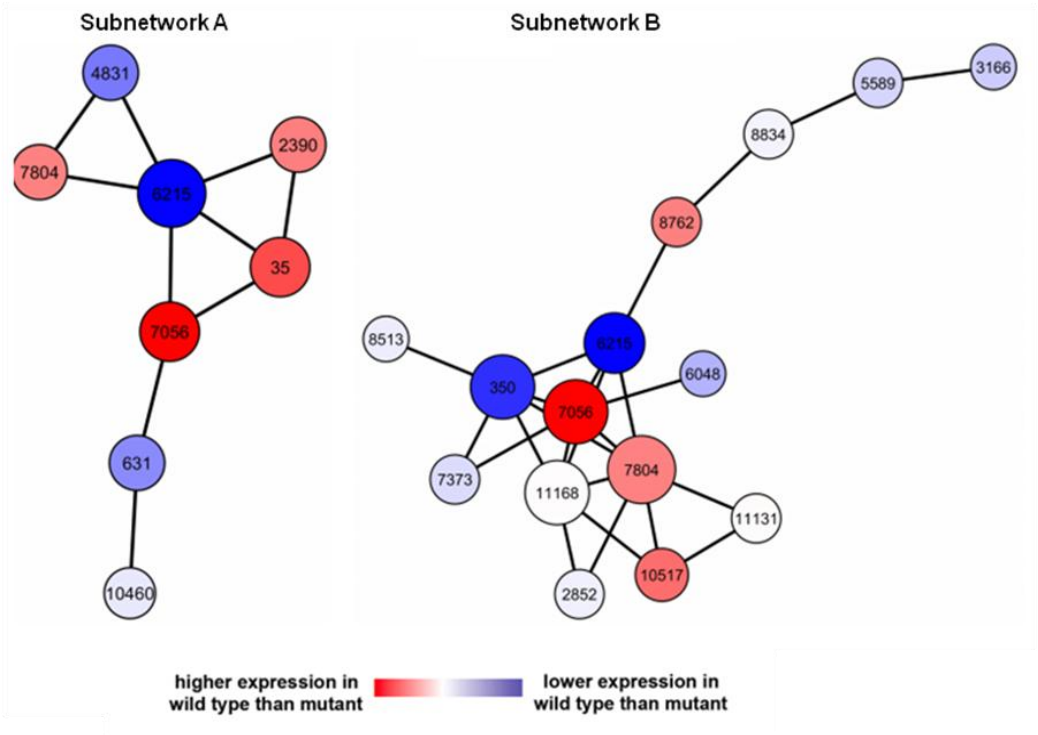


Figure 6. Predicted subnetwork modules. A network-based comparative analysis was used to compare the expression activity and correlation of secondary metabolite genes between *Mads1* and the WT datasets.

3.2.3 In silico *subnetwork module analysis and gene selection*

The two predicted subnetworks were analyzed *in silico* and putative hub genes were selected based on the following criteria: i) increased expression in the WT compared to *Mads1*, ii) putative protein domain, iii) number of paralogs in the genome, iv) yeast orthologs, and v) known function in yeast or other fungal species. The putative protein domain and number of paralogs in the *F. verticillioides* genome was predicted using the Broad Institute's Fusarium Comparative Database (www.broadinstitute.org) and NCBI's TBLASTN algorithm (blast.ncbi.nlm.nih.gov). Information on yeast homology and putative function in *S. cerevisae* was collected through the *Saccharomyces* Genome Database (www.yeastgenome.org). A promoter binding site prediction analysis was performed using SCOPE and the recognized MADS-box Serum Response Factor 1 (*SRF1*) CArg-box binding motif, $CC[A/T]_6GG$, as a potential target (Chakravarty et al. 2007). SCOPE applies three types of algorithms, *PRISM, BEAM,* and *SPACER*, to determine putative transcription factor binding sites up to 1 kb upstream of the gene of interest.

3.2.4 Nucleic acid manipulation and quantitative real-time RT-PCR (qRT-PCR)

Fungal genomic DNA was extracted using a phenol:chloroform:isoamyl alcohol extraction as previously described (Majumdar et al. 1991). Total RNA was extracted using the Qiagen RNAeasy Mini kit (Qiagen) following the manufacturer's protocol. Following a two-step approach, mRNA was converted into cDNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific) following the manufacturer's protocol.

The qRT-PCR gene expression analyses were performed on an Applied Biosystems QuantStudio 6 Real Time PCR system using the SYBR Green Dynamo ColourFlash qPCR kit (Thermo Fisher Scientific). A 0.5 µl cDNA reaction was applied as the template and the *F. verticillioides* β-tubulin-encoding gene (*TUB2*) was used as the endogenous calibrator.

3.2.5 Fusarium verticillioides *molecular manipulation*

Gene-deletion mutants were generated in the WT strain (7600) via homologous recombination with split marker gene-disruption constructs (Fu et al. 2006). Partial fragments of the hygromycin B phosphotransferase gene (*HPH*) designated HP (766 bp) and PH (924 bp) were fused to the flanking regions of the gene of interest via joint-PCR. *F. verticillioides* protoplasts were prepared and transformed as previously described (Sagaram and Shim 2007). Transformants were grown and selected on regeneration media containing Hygromycin (50 mg/L). Polymerase chain reaction (PCR) and/or Southern hybridization (Sambrook et al. 1989) were used to verify the putative transformant genotypes.

3.2.6 Conidia production, vegetative growth, and microscopy

V8 agar, defined liquid (DL) medium with agar (Shim and Woloshuk 2001), and 0.2% potato dextrose agar (PDA) plates were inoculated with a 20 µl ($1x10^6$) spore suspension and incubated at 25°C with a 14 h light/10 h dark cycle. Four biological

replicates were used for the vegetative growth and conidia production experiments. For the initial phenotypic assay, Sng1, Sng2 and the WT radial colony growth was measured and recorded at 6-dpi. A student's t-test was used to determine statistical significance based on a 95% confidence level. Later, the Sng3, Sng4, Sng5, and WT strains were inoculated onto the three media types to assess the radial colony growth rate. I started measuring and recording radial colony growth at 2-dpi and continued daily for up to 6-dpi. Statistical significance was determined based on a 95% confidence interval using a one-way Analysis of Variance (ANOVA). For conidia production analysis, 2-ml of sterile water was added to each V8 plate and conidia were resuspended by scraping the colony surface with a sterile spreader. Conidia were enumerated using a haemocytometer. A student's t-test was used to determine statistical significance based on a 95% confidence level.

Fungal strains were grown on V8 medium for morphological study. All image acquisition was performed using an Olympus BX51 fluorescence microscope outfitted with an Olympus DSU (http://www.olympus-ims.com/en/microscope/) and a Hamamatsu Orca ER camera (http://sales.hamamatsu.com/). The agar block method was used to mount slides for microscopic observation.

3.2.7 FB_1 assay

For FB_1 production analyses, WT and mutant fungal tissue was grown in defined liquid (DL) medium at 25°C on a 150-RPM rotary shaker (Shim and Woloshuk 2001). Approximately 2 ml of the culture supernatant containing excreted FB_1 was harvested at

5- and 7-dpi. For purification, 1 ml of FB_1 crude extract was passed through an equilibrated HyperSep C18 SPE column (Thermo Fisher Scientific) as previously described (Christensen et al. 2012). High Performance Liquid Chromatography (HPLC) analysis of FB_1 was performed as described previously (Shim and Woloshuk 1999). FB_1 levels were normalized based on the dry mass of fungal mycelia. The experiment was conducted with at least three biological replicates. A student's t-test was used to determine statistical significance based on a 95% confidence level. FB_1 production experiments in the Sng1/Sng2 and the Sng3/Sng4/Sng/5 gene-deletion mutants were conducted separately.

3.3 Results

3.3.1 Network based comparative analysis and putative hub gene identification

The two proposed subnetworks were analyzed *in silico*, and five genes were selected as candidates for functional characterization (Figure 7). Hub gene prediction was based on five criteria, including putative function and homology to other known fungal genes in secondary metabolism. FVEG_00035, FVEG_07056, FVEG_02390, and FVEG_07804 were selected from subnetwork A and are all upregulated in the WT compared to *Mads1* gene-deletion mutant (Table 2a). FVEG_07804, FVEG_07056, and FVEG_11168, a constitutively expressed gene, were selected from subnetwork B (Table 2b). While the majority of the genes identified are differentially regulated downstream of MADS-box TFs, the putative CArg-box binding motif, $CC[A/T]_6GG$, was not

identified in the 1 kb promoter regions by SCOPE algorithms, suggesting that MADS-box TFs do not directly bind to these genes in *F. verticillioides.*

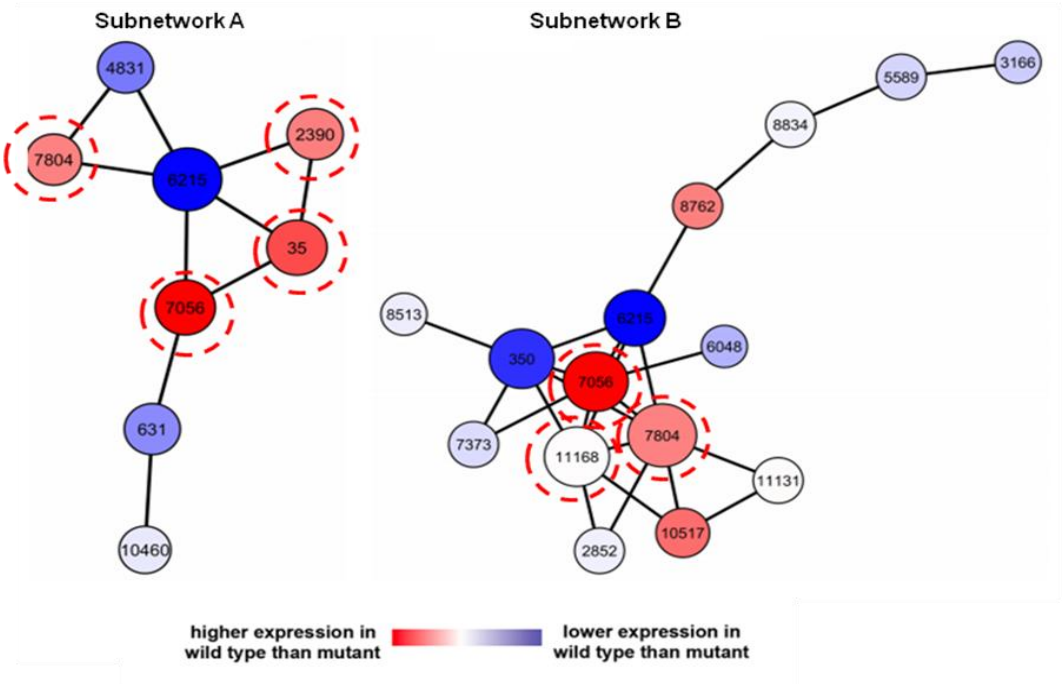


Figure 7. Subnetwork genes selected for functional characterization. Based on the *in silico* analysis, five genes were predicted as putative hub genes and selected for functional characterization (circled in red).

Gene	Protein domain prediction	# of Paralogs	Yeast Homolog	Known Function
FVEG_00035	50S Ribosome-binding GTPase		NOG1	Required for 60s ribosome sub-unit biogenesis in yeast
FVEG_02390	RAS GTPase	1 copy	RSR1, RAS2, RAS1, GSP1, GSP2	GTP-binding protein of the Ras superfamily; required for bud site selection, morphological changes in response to mating pheromone, and efficient cell fusion
FVEG_07056	Homeobox KN Domain	4 copies	CUP9, TOS8	Homeodomain-containing transcriptional repressor; regulates expression of PTR2, which encodes a major peptide transporter; imported peptides activate ubiquitin-dependent proteolysis, resulting in degradation of Cup9p and de-repression of PTR2 transcription; protein abundance increases in response to DNA replication stress
FVEG_07804	Cyanase_C (cyanate lyase)	1 copy	None	Unknown

Table 2a. Candidate genes for functional characterization in Subnetwork A.

Gene	Protein domain prediction	# of Paralogs	Yeast Homolog	Known Function
FVEG_11168	Methyltransferase	1 copy	EFM3, NNT1, YNL024C	S-adenosylmethionine-dependent methyltransferase; seven-beta-strand lysine methyltransferase which trimethylates translation elongation factor EF2 (Eft1p and Eft2p)
FVEG_07804	Cyanase_C (cyanate lyase)	1 copy	None	Unknown
FVEG_07056	Homeobox KN Domain	4 copies	CUP9, TOS8	Homeodomain-containing transcriptional repressor; regulates expression of PTR2, which encodes a major peptide transporter; imported peptides activate ubiquitin-dependent proteolysis, resulting in degradation of Cup9p and de-repression of PTR2 transcription; protein abundance increases in response to DNA replication stress

Table 2b. Candidate genes for functional characterization in Subnetwork B.

3.3.2 Generation of Subnetwork gene (Sng) deletion mutants

To perform the functional and phenotypic analysis of selected putative hub genes, gene-deletion mutants, Sng1 (ΔFVEG_00035::HPH) and Sng2

(ΔFVEG_07056::HPH), were generated in the WT strain (Table 1). The putative mutant strains were first screened using polymerase chain reaction (PCR) (data not shown), and then subsequently confirmed by Southern Hybridization (Sambrook et al. 1989) (Figure 8a). Later, three additional gene-deletion mutants, Sng3 (ΔFVEG_02390::HPH), Sng4 (ΔFVEG_07804::HPH), and Sng5 (ΔFVEG_11168::HPH), were generated in the WT strain (7600). Putative mutants were screened and confirmed by polymerase chain reaction (PCR) (Figure 8b).

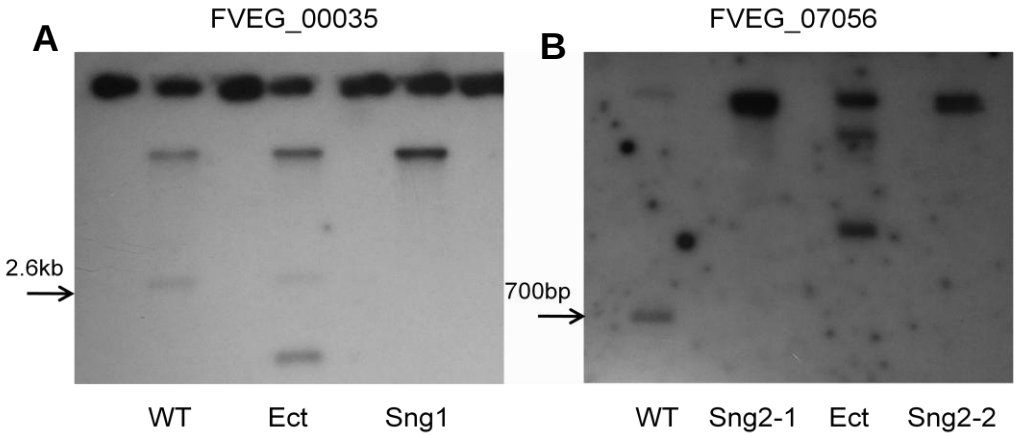


Figure 8a. Verification of Sng1 and Sng2 putative gene-deletion mutants with Southern Hybridization. Genomic DNA was extracted from the WT and putative gene-deletion mutants, Sng1 and Sng2, and then cut with restriction enzyme. P^{32} was used as a probe to hybridize the left flank construct onto the blot. A) A 2.6 KB band was expected in the WT but not in Sng1. B) A 700 BP band was expected in the WT but not in Sng2.

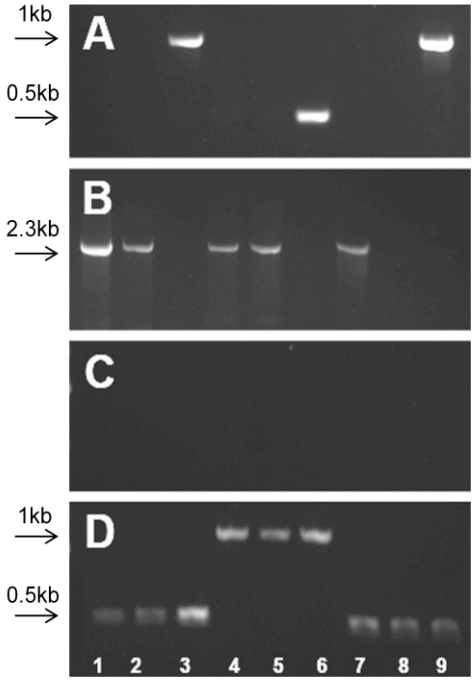


Figure 8b. Verification of Sng3, Sng4, and Sng5 putative gene-deletion mutants with polymerase chain reaction (PCR). *F. verticillioides* genes, FVEG_02390, FVEG_07804, and FVEG_11168 were targeted for gene-deletion by homologous recombination. Putative mutants were verified by PCR. A. Inside open reading frame (ORF) amplification screen; B. Amplification of 500-bp upstream plus the right flank downstream primer of hygromycin (YG/F); C. negative controls; D. positive controls. 1: Sng3-1, 2: Sng3-2, 3: WT, 4: Sng4-1, 5: Sng4-2, 6: WT, 7: Sng5-1, 8: Sng5-2, and 9: WT.

3.3.3 Phenotypic analysis

The WT and gene-deletion mutants were analyzed for growth rate on PDA, V8, and synthetic Defined Liquid (DL) medium with agar (Shim and Woloshuk 2001). There was a significant reduction in radial colony growth between the WT and Sng2 and Sng1 strains on DL medium and 0.2% PDA medium, respectively (Figure 9). No

significant difference in the radial colony growth rate was detected between the WT, Sng3, Sng4, and Sng5 strains on the three media tested (Figure 10). All knockout mutants showed similar phenotypes on solid culture when compared to that of the WT, although aerial hyphae were slightly reduced (data not shown). Conidia generation was enumerated after 6-dpi on V8 medium. There was no significant difference in conidia production between the WT, Sng4, and Sng5 mutants (Figure 10). However, Sng3 generated significantly higher conidia when compared to the WT. Microscopic analysis revealed no difference in hyphal or conidia development (data not shown). Pigmentation of Sng1, Sng2, Sng3, and Sng5 cultures growing in Defined Liquid (DL) medium was found to be altered at 7-dpi (Figure 11).

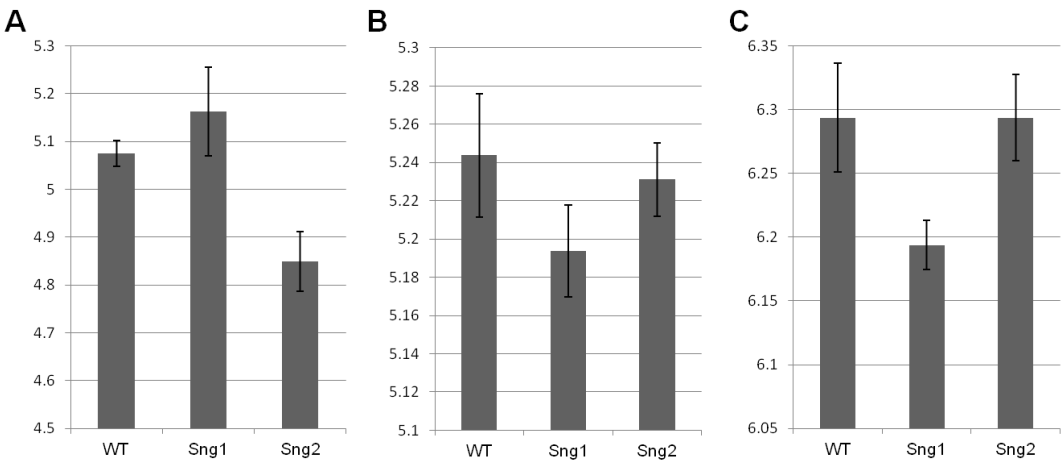


Figure 9. Growth rate analysis of the WT, Sng1, and Sng2 strains on various media. Mycelial growth was measured in WT, Sng1 and Sng2 on various media after 6-dpi. A) Growth rate on Defined Solid medium, B)

Growth rate on Potato Dextrose Agar (PDA) medium, C) Growth rate on V8 medium. Error bars indicate standard error between biological replicates.

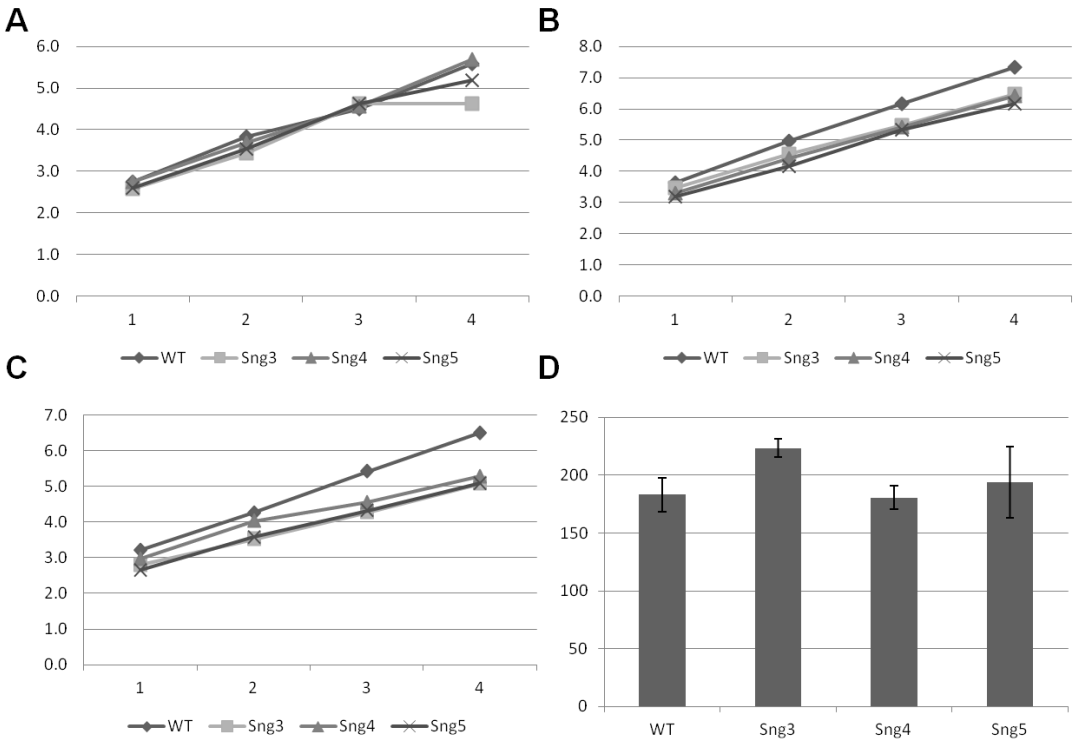


Figure 10. Growth rate and conidia production analysis of the WT, Sng3, Sng4, and Sng5 strains on various media. Mycelial growth and conidia generation was measured in WT, Sng3, Sng4 and Sng5 on various media from 2-dpi to 6-dpi. A) Growth rate on Potato Dextrose Agar (PDA) medium, B) Growth rate on V8 medium, C) Growth rate on Defined Solid medium. D) Conidia enumeration on V8 medium at 6-dpi. Error bars indicate standard error between biological replicates.

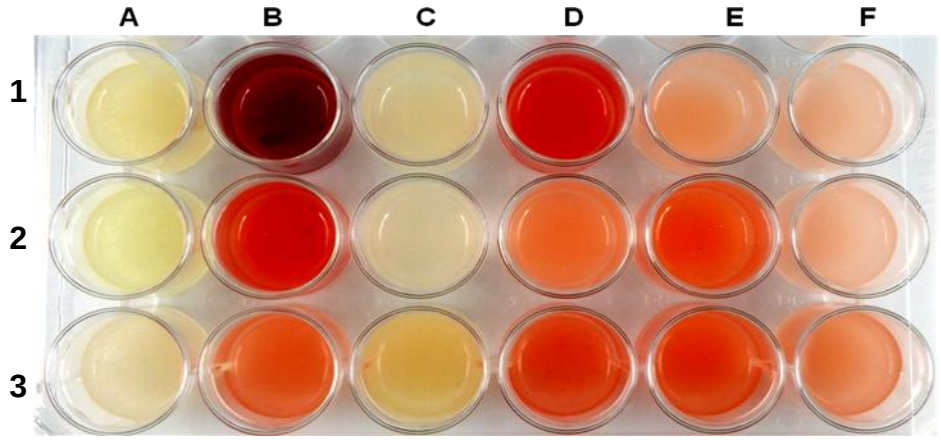


Figure 11. Phenotypic profile of WT and gene-deletion mutants in Defined Liquid (DL) medium. Pigmentation was altered between the WT and Sng gene-deletion mutants when grown in Defined Liquid (DL) medium at 7-dpi. Column: A) WT, B) Sng3, C) Sng4, D) Sng5, E) Sng1, F) Sng2. Row numbers indicate biological replicate per fungal strain.

3.3.4 FB_1 production in synthetic liquid culture

The WT and gene-deletion mutant strains were inoculated into DL media and analyzed for FB_1 production at 5- and 7-dpi (Figure 12). Sng1 produced significantly higher concentrations of FB_1 at 7-dpi. There was no significant difference in the production of FB_1 in the Sng2 strain compared to the WT. Sng3, Sng4 and Sng5 were analyzed for FB_1 biosynthesis in a later experiment. All three strains were found to have significantly reduced FB_1 biosynthesis in comparison to the WT at both 5- and 7-dpi.

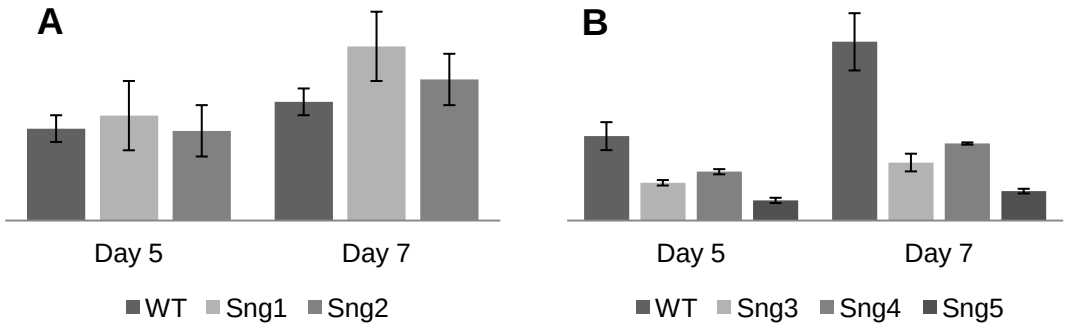


Figure 12. Relative FB_1 Production of the WT versus gene-deletion mutants. The WT and gene-deletion mutants were cultured in Defined Liquid (DL) medium for 5- and 7-dpi. The supernatant was collected, filtered, and analyzed for FB1 using High Performance Liquid Chromatography (HPLC). A) Relative FB1 production in the WT compared to the Sng1 and Sng2 gene-deletion mutants at 5- and 7-dpi. B) Relative FB1 production in the WT compared to the Sng3, Sng4, and Sng5 gene-deletion mutants at 5- and 7-dpi. Error bars indicate standard error of biological replicates.

3.3.5 Quantitative RT-PCR subnetwork gene expression analysis

Gene expression within the two subnetworks was analyzed to test the network robustness (Figure 13). Gene-deletion mutants, Sng3 (FVEG_02390) and Sng 5 (FVEG_11168) resulted in the greatest reduction in FB_1 biosynthesis, and subsequently hypothesized as putative hub genes. In theory, mutation of the hub gene would alter expression of neighboring genes within the network or result in a complete network failure. In subnetwork module A, expression of FVEG_06215 was greater than 2-fold higher in WT versus Sng3 at 5-dpi, but differential expression was negligible at 7-dpi (Figure 14). Preliminary results also revealed a negligible difference in the expression

of FVEG_00035 at 5-dpi in the Sng3 strain compared to the WT, however transcript levels increased by approximately 9-fold at 7-dpi. At 5-dpi FVEG_07056 was expressed over 2-fold higher in the WT versus Sng3, and then increased by a difference of over 100-fold at 7-dpi.

Gene expression analysis of Subnetwork module B revealed a 2-fold decrease in the expression of FVEG_07056 in the WT compared to the Sng5 mutant strain at 5-dpi (Figure 14). Expression of FVEG_07056 continued to decrease in the WT, with a greater than 4-fold decrease in the number of transcripts by 7-dpi. Differential expression was negligible in the WT compared to the Sng5 mutant at 5-dpi, but transcripts decreased in the WT by over 2-fold on 7-dpi. Neighboring genes of Sng1, Sng2, and Sng4 were also analyzed to determine broader changes of expression within subnetworks, as well as to infer regulatory directionality (Figure 15).

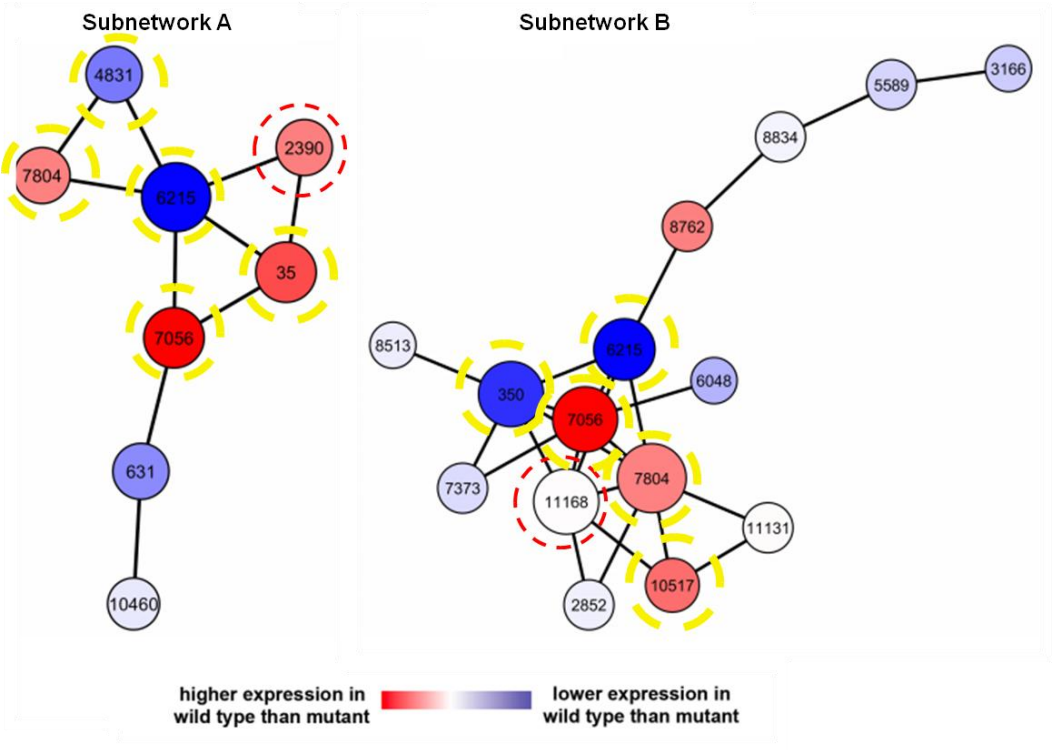


Figure 13. FVEG_02390 and FVEG_11168 neighboring subnetwork genes selected for quantitative real-time RT-PCR (qRT-PCR) analysis. FVEG_02390 and FVEG_11168 are circled in red; the genes selected as targets for expression analysis are circled in yellow.

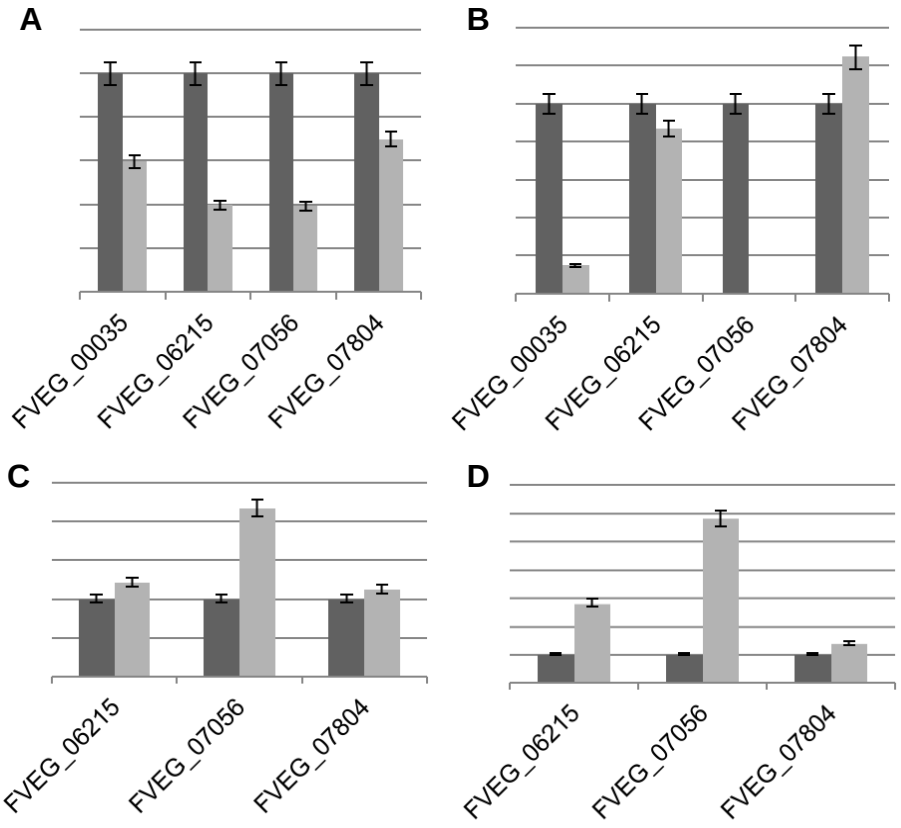


Figure 14. Subnetwork gene expression analysis of WT vs. Sng3 and Sng5 gene-deletion mutants. The relative transcript quantity of subnetwork genes was analyzed in the WT vs. Sng3 and Sng5 gene-deletion mutants at 5- and 7-dpi in Defined Liquid (DL) medium using qRT-PCR. Trascripts were normalized based on WT β-tubulin levels. A) Relative transcript quantity of subnetwork A genes in the WT vs. Sng3 at 5-dpi. B) Relative transcript quantity of subnetwork A genes in the WT vs. Sng3 at 7-dpi. C) Relative transcript quantity of subnetwork B genes in the WT vs. Sng5 at 5-dpi. D) Relative transcript quantity of subnetwork B genes in the WT vs. Sng5 at 7-dpi. Error bars indicate standard error of biological replicates.

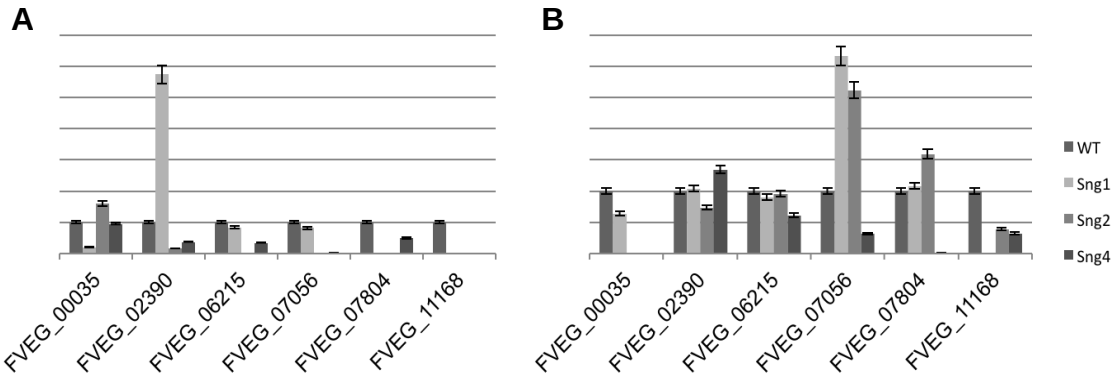


Figure 15. Subnetwork gene expression analysis of WT vs. Sng1, Sng2, and Sng4 gene-deletion mutants. The relative transcript quantity of subnetwork genes was analyzed in the WT vs. Sng1, Sng2 and Sng4 gene-deletion mutants at 5- and 7-dpi in Defined Liquid (DL) medium by qRT-PCR. Transcripts were normalized based on WT β-tubulin levels. A) Relative transcript quantity of subnetwork A and B genes in the WT vs. Sng1, Sng2 and Sng4 at 5-dpi. B) Relative transcript quantity of subnetwork A and B genes in the WT vs. Sng1, Sng2 and Sng4 at 7-dpi. Error bars indicate standard error of biological replicates.

3.4 Discussion

The use of transcriptomics to identify important novel genes involved in growth and development, primary and secondary metabolism, reproduction, and pathogenicity, have become increasingly popular over the last decade. However, the traditional method of analyzing differential expression as a single-gene-focused approach is limiting and can be especially challenging when mining large data sets. In this research, a systematic approach was used to predict networks of genes that act downstream of MADS-box TFs with an impact on secondary metabolite and mycotoxin production in *F. verticillioides*.

Two subnetworks were predicted based on six criteria. An *in silico* analysis was performed to validate the biological relevance of genes within subnetworks, and to gain insight into the predicted function of each gene. Five putative hub genes were selected from each subnetwork for phenotypic characterization.

Bioinformatics analysis revealed that the selected genes likely play a role in signal transduction, secondary metabolism, and/or maize pathogenicity. *F. verticillioides* genes, FVEG_00035 (Sng1) and FVEG_02390 (Sng3) were found to encode a 50S ribosome-binding GTPase and a RAS GTPase, respectively. GTPase superfamily proteins which bind and hydrolyze GTP are known to be involved in a diverse array of cellular functions, including signal transduction and ribosomal biosynthesis (Bourne et al. 1990; Khosravi-Far and Der 1994). FVEG_07056 (Sng2) and FVEG_11168 (Sng5) also encode for genes associated with regulatory and signaling pathways in both prokaryotic and eukaryotic organisms. Homeodomain protein, *HTF1*, was found to influence asexual reproduction and conidiophore generation in *M. oryzae* (Liu et al. 2010). Methyltransferase domains, such as those encoded by some *PKS* genes and regulatory protein, *e.g.* LaeA, play significant roles in secondary metabolism through the methylation of heterochromatic and euchromatic regions of eukaryotic chromosomes (Keller et al. 2005; Lee et al. 2005). The final gene identified, FVEG_07804 (Sng4), was found to be differentially regulated in both subnetworks and encodes for a cyanate lyase domain, a recently emerged, novel enzyme capable of detoxifying plant-produced cyanate (Elmore et al. 2015). Little information is available on the functional capacity of cyanate lyase genes in secondary metabolite production or

host-pathogen interactions. It is also worth noting that each set of subnetwork genes was also analyzed with the SCOPE promoter binding prediction software, although failed to predict the known MADS-box TF CArg-box binding motif, (CC[A/T]$_6$GG)(Shore and Sharrocks 1995; Chakravarty et al. 2007). This indicates that the differentially expressed genes found in the predicted subnetworks are activated further downstream and are not directly regulated by MADS-box TFs.

All five mutations resulted in a change in secondary metabolism, with four of the gene-deletion strains leading to a significant difference in FB_1 production versus the WT. FB_1 biosynthesis was deficient in the Sng3, Sng4, and Sng5 mutants, conversely, the Sng1 mutant produced significantly higher concentrations of FB_1 compared to WT in DL medium. Gene-deletion in Sng3, Sng4, and Sng5 did not significantly suppress radial colony growth rate or conidiogenesis on various media, suggesting that the reduction in FB_1 biosynthesis was not attributable to decreased fitness. Pigmentation of Sng1, Sng2, Sng3, and Sng5 inoculum was also modified in DL medium, indicating supplemental effects on polyketide synthase (PKS) regulation and transcription. Along with fumonisin biosynthesis, polyketide synthases (*PKS*) are also associated with other secondary metabolic products generated by fungal pathogens, such as pks-4 in *Trichoderma reesei,* pks-1 in *Chaetomium globsum* and fusarubin in *F. fujikuroi* (Hu et al. 2012; Studt et al. 2012; Atanasova et al. 2013). These results provide evidence to support that the network-based comparative analysis can be applied to improve identification of important novel genes that directly influence secondary metabolism, and otherwise may not have been recognized using the traditional single-gene approach.

Once the putative hub genes were targeted for gene deletion, the next question raised was whether the expression pattern in these subnetworks, particularly the neighboring genes, are altered in these new mutants. Quantitative real-time RT-PCR (qRT-PCR) analysis was used in an effort to answer this question and to provide insight into the relationship between coordinated gene expression influencing secondary metabolism. The RAS GTPase (Sng3, ΔFVEG_02390) and methyltransferase (Sng5, ΔFVEG_11168) gene-deletion mutants had the greatest reduction in FB_1 biosynthesis compared to the WT, so I focused on these genes for subsequent qRT-PCR analysis. In subnetwork A, FVEG_02390 shares a direct link to FVEG_06215 and FVEG_00035. Expression of FVEG_06215 and FVEG_00035 were found to be significantly higher in the WT compared to the Sng3 mutant. Interestingly the greatest expression difference between Sng3 and the WT occurred in FVEG_07056, a gene separated from FVEG_02390 by both FVEG_06215 and FVEG_00035 in the subnetwork. When I analyzed the differential expression of FVEG_00035 and FVEG_06215 in the Sng2 (ΔFVEG_07056), there was no significant difference at either time point. These results suggest that overall network robustness was found be substantially altered in Sng3 when compared to the WT strain, demonstrating a possible hub role for FVEG_02390 in subnetwork A. The proposed 'hub' role for FVEG_02390 is further supported by what is known about the functional importance of RAS GTPases in various cellular processes. RAS GTPases have been identified as broad transcriptional regulators in both prokaryotic and eukaryotic systems (Wuichet and Søgaard-Andersen 2015). As stated earlier, RAS GTPases hydrolyze and bind to GTP, subsequently acting as a "on-off"

switch in signal transduction pathways (Bourne et al. 1990). For example, *Beauveria bassiana* RAS GTPase protein, Ras3, was found to act upstream of the Hog1 signaling pathway by modulating conidiation, multi-stress tolerance and virulence (Guan et al. 2015). In *T. reesei,* TrRas1 plays major roles in growth and development, while TrRas2 transcriptionally regulates cellulase gene expression, ultimately providing evidence of RAS GTPase requirements in secondary metabolite production (Zhang et al. 2012).

Subnetwork B is considerably more complex and therefore presented additional challenges when testing for transcriptional network robustness. The methyltransferase encoding gene, FVEG_11168, is constitutively expressed in the WT and *Mads1,* and shares edges with five other genes in the subnetwork. Preliminary qRT-PCR analysis revealed a similar expression pattern of FVEG_07056 and FVEG_06215, with a significant transcript reduction in the WT compared to Sng5. It could be inferred that the FVEG_11168 methyltransferase may be associated in the pathway that acts to suppress the expression of FVEG_07056 and therefore FVEG_06215. This would also explain the inverse expression results found in the Sng2 mutant. This functional prediction is not completely unfounded, as methyltransferases have been previously reported to suppress gene expression in other eukaryotes, and is perhaps best studied in animal carcinogenesis (Mukai and Sekiguchi 2002). Evidence for methylation-induced gene-silencing has also been shown in fungal systems, such as *SET1* in *S. cerevisiae* (Briggs et al. 2001). Deletion of *SET1* was found to completely abolish histone H3 Lys4 methylation *in vivo*, thereby leading to the repression of RNA polymerase II transcription. Furthermore, methyltransferase Llm1, was found to suppress T-toxin

biosynthesis in *Cochliobolus heterostrophus (Bi et al. 2013).* L1m1 gene-deletion mutants also led to an alteration in pigmentation, providing additional evidence for a role in secondary metabolite production. An alternative hypothesis is that the relationship between FVEG_11168 and FVEG_07804 is influenced by FVEG_07804 since differential expression occurred primarily in Sng4 but not in Sng5. However since little is known about the function of cyanase in fungal secondary metabolism, it is difficult to predict the role of FVEG_11168 in this subnetwork before we investigate the expression profile of other genes correlated in subnetwork B.

4. CONCLUSIONS AND FUTURE WORK

Next generation sequencing (NGS) is dramatically changing the way researchers study transcriptomics and signal transduction pathways during various stages of an organism's lifecycle. However NGS is not without its challenges, and there is now a growing demand for the development of improved approaches to analyze the massive datasets generated (Ozsolak and Milos 2011). In this study, a computational network-based comparative analysis was successful in identifying important subnetwork modules downstream of MADS-box TFs that are predicted to be associated with secondary metabolism in *F. verticillioides.* Five genes were characterized from two predicted subnetworks and two gene-deletion mutations resulted in a dramatic reduction in FB_1 biosynthesis. In addition to functional characterization, I also attempted to test the hypothesis on the transcriptional regulation directionality of the genes in the subnetworks. Unfortunately, some questions remain unanswered and further study into the expression patterns of genes within the subnetworks, including the downstream effects on the *PKS* expression, is needed. In addition, complementation strains are needed to verify phenotypic characteristics. This study also identified a novel enzyme, cyanase, that plays a significant role in FB_1 biosynthesis in *F. verticillioides.* Continued phenotypic characterization of the Sng4 mutant may provide insight into functional significance of cyanase in secondary metabolite production and pathogenesis. Sng4 will be further tested for virulence and FB_1 production under nitrogen-limiting conditions in an attempt to answer some of these questions. Ongoing research into the prediction of

signal transduction pathways using NGS data and emerging computational approaches will continue to expand our knowledge of *F. verticillioides* - host interactions.

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